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VALIDATION OF A RT-qPCR ASSAY FOR DIFFERENTIAL DIAGNOSIS OF AFRICAN AND CLASSICAL SWINE FEVER

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Abstract. *A real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) assay for differential diagnosis of African and Classical swine fever has been developed in the Institute of Veterinary Medicine of the NAAS. The proposed assay allows simultaneous detection of three targets: ASFV, CSFV and an internal control, which helps to save time and money. In this article, an inter-laboratory validation of the developed RT-qPCR assay was described. Validation of the test kit was performed in compliance with OIE Validation Guidelines according to analytical sensitivity, specificity and repeatability. The limit of detection (LOD) of the validated RT-qPCR assay was 10 copies of the ASFV and CSFV genomes, respectively. Repeatability of the assay was shown to be high enough. The specificity of the assay, evaluated on different viral strains showed no cross-reactions with closely related pathogens (porcine circovirus type 2, porcine reproductive and respiratory syndrome virus and virus of Aujeszky's disease). The expected reactivity for ASFV and CSFV were observed. In conclusion, all investigated analytical performance criteria of the validated RT-qPCR assay for differential diagnosis of ASF and CSF are in compliance with international standards, which ensures accurate and definite results. This laboratory assay will be a valuable tool for rapid differential diagnosis in case of swine fever suspected outbreaks.*

Keywords: *African swine fever (ASF), Classical swine fever (CSF), RT-qPCR, validation.*

African swine fever (ASF) and Classical swine fever (CSF) are two transboundary contagious viral swine diseases with high mortality rates and huge economic impacts [2, 7]. There is a significant difference between ASF and CSF control and prevention measures mainly due to the presence of vaccines against CSF and their absence against ASF [3, 4]. Therefore, in the case of a suspected outbreak, differential diagnosis of the two diseases is essential. ASF cannot be differentiated from CSF by either clinical presentation or post mortem examination. For this reason laboratory diagnostic methods are useful only [5, 8].

The above listed points clearly indicate that the rapid and robust diagnosis of ASF and CSF is extremely important. That is why a single-step, real-time reverse-transcriptase polymerase chain reaction (RT-qPCR) assay for the simultaneous and differential laboratory diagnosis of Classical swine fever virus (CSFV) and African swine fever virus (ASFV) has been developed in the Institute of Veterinary Medicine of the NAAS [7].

The goal of the work was to provide inter-laboratory validation of the developed RT-qPCR assay for the simultaneous and differential CSFV and ASFV detection.

Materials and methods. The study was carried out in the Research Training Center For Animal Disease Diagnostics of the IVM NAAS.

Samples. In the study, we used control samples: ASF and CSF positive (PC ASF+CSF), negative (NC) and an exogenous internal control (IC). Plasmids carrying specific ASF and CSF sequences (1.0×10^5 copies/cm^3), pathological materials with ASFV, blood samples with CSFV – strain "Washington", cell cultures containing closely related viruses (porcine circovirus type 2 (PCV-2), porcine reproductive and respiratory syndrome (PRRS) virus and virus of Aujeszky's disease) were used for validation of the assay.

DNA/RNA extraction. «RIBO-Sorb» (AmpliSens) was used for DNA/RNA extraction, according to the manufacture’s instruction. During the nucleic acid

extraction, 10 µl of IC DNA was added into each sample suspended in the lysis buffer.

Amplification. Single-tube PCR reactions were prepared pooling 17.0 µl of PCR-mix and 3.0 µl of Primer-mix. Finally, a 5.0 µl aliquot of DNA/RNA extractions from the samples were added to 20 µl of PCR master mix.

Amplification was performed using Rotor-Gene Q («QIAGEN Hilden»). The cycling protocol was as follows:

1. Thermocycling conditions for the RT-qPCR

№	Steps	Temperature, ºC	Time	Cycles
1	Reverse transcription	50	30 min	1
2	Enzyme activation	95	10 min	1
2	Denaturation	95	20 sec	45
	Primer annealing	58 *	20 sec	
	Elongation	72	30 sec	

* Fluorescence values were collected at 58 °C in three channel: FAM (DNA of ASFV), ROX (cDNA of CSFV) and JOE (IC).

Interpretation of results. The results can be considered valid if: the Ct-value of PC ASF+CSF ≤ 35 on the FAM and ROX channels, the Ct of NC – absent, the Ct of IC of all investigated samples ≤ 35 on the JOE channel. The sample is ASF positive if the Ct-value ≤ 40 on the FAM channel and CSF positive if the Ct ≤ 40 on ROX. The sample is negative if Ct-values are absent on the FAM and ROX channels but on JOE Ct ≤ 35.

Validation. Validation of the test kit was performed in compliance with OIE Validation Guidelines 2014 – 3.6.3 «Development and optimization of Nucleic acid detection assays» [1].

Results. The diagnostic method was validated according to such analytical performance criteria as sensitivity, specificity and repeatability.

The analytical sensitivity of the assay were determined by testing 10-fold serial dilutions of the two quantified plasmids carrying specific ASFV and CSFV sequences respectively (from 1.0×10^5 to 1.0×10^0 viral copies per reaction) in DNAse-free water (Figure 1).

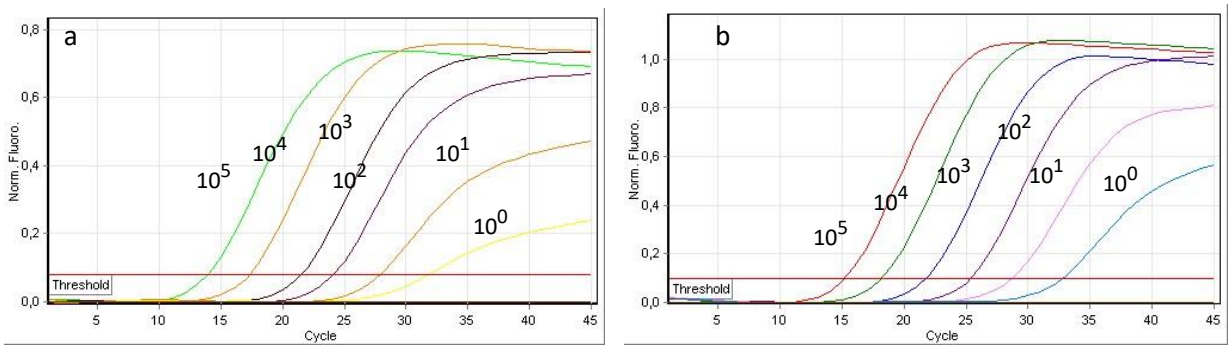

Figure 1. The amplification curves 10-fold serial dilution of the plasmids: a) carrying specific ASFV sequences (FAM channel); b) carrying specific CSFV sequences (ROX channel)

The lowest dilution detected with the Ct ≤40 was established as the limit of detection (LOD). The serial dilutions were examined in 10 independent assays (reproductions). Obtained results of ASFV and CSFV analytical sensitivity determination of the assay are presented in Tables 1 and 2.

Table 2 shows that the confidence interval of the analytical sensitivity of validated assay for ASFV was 100 % at concentration ranging from $1{,}0\times10^5$ to $1{,}0\times10^1$ copies/cm^3, and 30 % – at $1{,}0\times10^0$ copies/cm^3. Therefore, it was determined that LOD of the developed test system is at least 10 copies of the ASFV DNA.

2. Results of the 10-fold serial dilution amplification of the plasmid with incorporated specific ASFV sequence

Number of copies/cm^3		$1{,}0x10^5$	$1{,}0x10^4$	$1{,}0x10^3$	$1{,}0x10^2$	$1{,}0x10^1$	$1{,}0x10^0$
Ct-value on the FAM channel (10 repeats)	1	13.92	17.33	21.49	24.13	27.93	31.81
	2	13.99	17.27	21.60	24.07	28.11	-
	3	14.01	17.93	21.21	24.38	27.76	-
	4	13.89	17.45	21.51	24.24	27.81	32.55
	5	13.73	17.44	21.34	24.57	28.28	-
	6	14.08	17.38	21.55	24.17	27.91	-
	7	14.27	17.86	21.12	24.09	28.01	-
	8	13.87	17.41	21.66	24.13	27.54	-
	9	13.66	17.54	21.44	24.60	27.81	31.79
	10	13.92	17.33	21.70	24.35	27.69	-
Average Ct-value		13.93	17.49	21.46	24.27	27.89	32.05
SD		0.17	0.22	0.19	0.19	0.21	0.43
CV		1.22	1.26	0.89	0.78	0.75	1.34
CVv		3.59	2.89	2.33	2.06	1.79	1.56
positive/negative		10/10	10/10	10/10	10/10	10/10	3/7
Analytical sensitivity, %		100	100	100	100	100	30

The confidence interval of the analytical sensitivity for CSFV within concentration $1,0\times10^5$ – $1,0\times10^1$ copies/cm^3 was 100 % and 30 % – at concentration $1,0\times10^0$ – 40 % (Table 3). LOD of the validated RT-qPCR assay was 10 copies of the CSFV genome.

3. Results of the 10-fold serial dilution amplification of the plasmid with incorporated specific CSFV sequence

Number of copies/cm^3		$1,0x10^5$	$1,0x10^4$	$1,0x10^3$	$1,0x10^2$	$1,0x10^1$	$1,0x10^0$
Ct-value on the ROX channel (10 repeats)	1	15.25	18.26	21.76	25.40	28.76	32.82
	2	15.26	18.45	21.53	25.17	28.99	-
	3	15.37	18.23	21.23	25.38	28.87	32.65
	4	15.53	18.15	21.67	25.04	28.71	-
	5	15.48	18.31	21.04	25.24	28.58	33.38
	6	15.24	18.38	21.61	24.87	28.92	-
	7	15.29	18.25	21.28	25.41	28.43	-
	8	15.24	18.47	21.66	25.12	28.45	-
	9	15.17	18.26	21.09	24.96	28.69	32.43
	10	15.68	18.51	21.18	25.31	28.89	-
Average Ct-value		15.35	18.33	21.41	25.19	28.73	32.82
SD		0.16	0.12	0.27	0.19	0.20	0.41
CV		1.04	0.65	1.26	0.75	0.70	1.25
CVv		3.26	2.73	2.34	1.98	1.74	1.52
positive/negative		10/10	10/10	10/10	10/10	10/10	4/7
Analytical sensitivity, %		100	100	100	100	100	30

Repeatability of the assay was determined as a measure of agreement between results obtained by testing 10-fold serial dilutions of plasmids at 10 runs using several operators in one laboratory (Tables 1 and 2). Repeatability was expressed as a coefficient of variation (CV). The standard deviation (SD) and CV was calculated for each concentration of six plasmids dilutions. Obtained SD values were lower than the maximum acceptable value of the standard deviation for the method (SD≤0.5). The values of CV were also lower than the acceptable value of the coefficient of variation for the method (CVv). Therefore, repeatability of the assay was shown to be high enough, with coefficients of variation ranging from 0.65 to 1.34 %.

Analytical specificity (cross-reactivity) is a measure of the assay's ability to detect only the targets, in our case – ASFV, CSFV and IC. For analytical specificity determination the panel of samples, containing ASFV, CSFV or without was

prepared, moreover the cell cultures containing defined pathogens which cause similar to ASF and CSF clinical syndromes such as PCV-2, PRRS, Aujeszky's diseases were used. All biological materials, number of samples and values of crossing thresholds are summarized in Table 4. The samples were tested separately and in a mixture of "foreign agent + ASFV + CSFV" (1:1:1) in triplicate.

4. Analytical specificity of the validated RT-qPCR assay

№.	Biological material	Number of replicates	Ct FAM (ASF)	Ct ROX (CSF)	Ct VIC (IC)
1.	pathological material with ASFV – positive biological material (ASF PBM)	3	17.80±0.13	N/d	24.51±0.16
2.	pathological material without ASFV	3	N/d	N/d	23.21±0.29
3.	swine blood with CSFV (strain "Washington") – positive biological material (CSF PBM)	3	N/d	31.58±0.14	24.07±0.31
4.	swine blood without CSFV	3	N/d	N/d	24.08±0.22
5.	ASF PBM + CSF PBM (1:1)	3	18.22±0.39	31.53±0.16	24.27±0.29
6.	cell culture with PRRS virus (strain "Lelystad")	3	N/d	N/d	23.96±0.16
7.	cell culture with PRRS virus (strain "Lelystad") + ASF PBM + CSF PBM (1:1:1)	3	18.11±0.17	31.89±0.23	24.39±0.26
8.	cell culture with PCV-2 (straine "Stoon 1010")	3	N/d	N/d	24.05±0.10
9.	cell culture with PCV-2 (straine "Stoon 1010") + ASF PBM + CSF PBM (1:1:1)	3	17.96±0.23	32.00±0.22	24.19±0.37
10	cell culture with Aujeszky's diseases virus (straine "Petrikivskiy-2006")	3	N/d	N/d	23.91±0.22
11.	культура клітин, що містить вірус хв. cell culture with Aujeszky's diseases virus (straine "Petrikivskiy-2006") + ASF PBM + CSF PBM (1:1:1)	3	17.83±0.18	31,74±0.23	24.42±0.27

As result, the expected reactivity for ASFV and CSFV were observed and absence of cross-reactions for the other tested porcine viruses were confirmed. No amplification curves and Ct values on FAM and ROX were detected using nucleic acid extracted from samples with PRRSV, PCV-2 and ADV.

Conclusions. All investigated analytical performance criteria of validated PT-qPCR assay for differential diagnosis of African and classical swine fever are in compliance with international standards, which ensures accurate and definite results.

The limit of detection (LOD) of the validated test kit was 10 copies of the ASFV and CSFV genomes. High enough results of repeatability were achieved. There were confirmed absence of false results and cross-reactions with other porcine viruses. Therefore, this laboratory assay will be a valuable tool for rapid differential diagnosis in case of swine fever suspected outbreaks.

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ВАЛИДАЦИЯ ОТ-ПЦР ТЕСТ-СИСТЕМЫ ДЛЯ ДИФФЕРЕНЦИАЛЬНОЙ ДИАГНОСТИКИ АФРИКАНСКОЙ И КЛАССИЧЕСКОЙ ЧУМЫ СВИНЕЙ

С. С. Мандыгра, Л. М. Музыкина, Л. Н. Ищенко, Г. А. Коваленко, И. В. Галка, В. Г. Спиридонов, Н. П. Сытюк, С. А. Ничик, С. Д. Мельничук

Аннотация. *В Институте ветеринарной медицины НААН разработана тест-система для дифференциальной диагностики африканской и классической чумы свиней методом обратно-транскриптазной полимеразной цепной реакции (ОТ-ПЦР) в режиме реального времени. Предложенная методика позволяет одновременно в одной пробирке детектировать три мишени: вирус АЧС, вирус КЧС и внутренний контроль, что способствует экономии времени и средств. В статье представлены результаты проведения внутрилабораторный валидации разработанного ОТ-ПЦР диагностикума. Валидацию осуществляли в соответствии с международными требованиями, учитывая показатели чувствительности, специфичности и сходимости. Предел обнаружения (LOD) валидированного ОТ-ПЦР диагностикума составил 10 копий ДНК вируса АЧС и 10 копий кДНК вируса КЧС. Установлено достаточно высокую сходимость результатов. При исследовании специфичности не наблюдалось перекрестных реакций с штаммами возбудителей, вызывающих сходные с АЧС и КЧС симптомы (цирковирус свиней 2-го типа, вирус репродуктивно-респираторного синдрома свиней и болезни Ауески). В целом, исследованые аналитические показатели валидированной ОТ-ПЦР тест-системы для дифференциальной диагностики АЧС и КЧС соответствуют международным стандартам, что гарантирует получение точных и надежных результатов. Эта тест-систума станет ценным средством быстрой дифференциальной диагностики в случае возникновения подозрения на вспышку чумы свиней.*

Ключевые слова: *африканская чума свиней (АЧС), классическая чума свиней (КЧС), ОТ-ПЦР в режиме реального времени, валидация*

ВАЛІДАЦІЯ ЗТ-ПЛР ТЕСТ-СИСТЕМИ ДЛЯ ДИФЕРЕНЦІЙНОЇ ДІАГНОСТИКИ АФРИКАНСЬКОЇ ТА КЛАСИЧНОЇ ЧУМИ СВИНЕЙ

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Анотація. *В Інституті ветеринарної медицини НААН розроблено тест-систему для диференційної діагностики африканської та класичної чуми свиней методом зворотно-транскриптазної полімеразної ланцюгової реакції (ЗТ-ПЛР) у режимі реального часу. Запропонована методика дозволяє одночасно в одній пробірці виявляти три мішені: вірус АЧС, вірус КЧС і внутрішній контроль, що сприяє економії часу та коштів. У статті представлені результати проведення внутрішньолабораторної валідації*

розробленого ЗТ-ПЛР діагностикума. Валідацію здійснювали відповідно до міжнародних вимог за показниками чутливості, специфічності і збіжності. Межа виявлення (LOD) валідованого ЗТ-ПЛР діагностикума склала 10 копій ДНК вірусу АЧС і 10 копій кДНК вірусу КЧС. Встановлено достатньо високу збіжність результатів. При дослідженні специфічності не спостерігалось перехресних реакцій із штамами патогенів, що викликають подібні з АЧС і КЧС симптоми (цирковірус свиней 2-го типу, вірус репродуктивно-респіраторного синдрому свиней і хвороби Ауєскі). Вцілому, досліджені аналітичні показники валідованої ЗТ-ПЛР тест-системи для диференціальної діагностики АЧС та КЧС відповідають міжнародним стандартам, що гарантує отримання точних та надійних результатів. Ця тест-систума стане цінним засобом швидкої диференційної діагностики в разі виникнення підозри на спалах чуми свиней.

Ключові слова: *африканська чума свиней (АЧС), класична чума свиней (КЧС), ЗТ-ПЛР у режимі реального часу, валідація*